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(71) Applicant *(for all designated States except US)*: **NEEM BIOTECH LTD.** [GB/GB]; 71 Heol y Coed, Rhiwbina, Cardiff CF14 6HR (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **WILLIAMS, David, Michael** [GB/GB]; 17 Heol y Coed, Rhiwbina, Cardiff CF14 6HR (GB). **PANT, Chandra, Mohen** [GB/GB]; 65 King George V Drive, Heath, Cardiff CF14 4EF (GB).

(74) Agent: **NASH, David Allan**; Haseltine Lake, Imperial House, 15-19 Kingsway, London WC2B 6UD (GB).

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(54) Title: PROCESS FOR THE PRODUCTION OF ALLICIN

(57) Abstract: There is disclosed a method for preparing allicin which comprises the following steps: (a) mechanically treating a natural source of alliinase to release alliinase therefrom; (b) contacting the mechanically treated alliinase source with an aqueous solution of alliin containing alliin at a concentration greater than that found in raw garlic, whereby the alliin is enzymatically converted to allicin by the alliinase released from the alliinase source; and optionally (c) extracting the resultant allicin into a low boiling point non-polar organic solvent.

PROCESS FOR THE PRODUCTION OF ALLICIN

The present invention relates to a process for the production of allicin from alliin, and a method for the preparation of pure allicin.

Garlic and onions are members of the lily family. Many medical properties have been ascribed to garlic and onions and they have been used in folk medicine for thousands of years.

A large spectrum of medical properties has been ascribed to garlic, Allium Sativum, (Block E 1985) Sci. Am. 252(3): 114-119). In modern times the interest in the therapeutic properties of garlic has been revived, and it is the object of an increasing number of biochemical and clinical studies. An extensive review of the beneficial properties of garlic extracts may be found in WO99/21008.

Garlic preparations are commercially available in the form of garlic oil, extracts, pills or tablets. Usually the preparation procedures of such garlic preparations are unknown, and the composition and amount of their active ingredients are not defined.

The active principles present in garlic have been found to be sulphur-containing compounds. The principal component of a colourless oil obtained from steam distillates of garlic extracts was shown to be allicin, an unusual sulphur compound of formula $C_6H_{10}S_2O$ (thio-2-propene-1-sulfinic acid S-allyl ester) (Cavallito et al., (1944) J. Am. Chem. Soc. 66, 1944-1954). Allicin was found to be a chemically unstable, colourless to straw coloured liquid. This liquid is thought to be responsible for both the odour and much of the biological activity of garlic.

Although allicin is responsible for the smell of garlic, a garlic bulb exhibits little or no odour until it is cut or crushed. The intact garlic clove does not contain allicin but rather its odourless precursor

alliin (+)(S-allyl-L-cysteine sulfoxide). This is converted to allicin, pyruvate and ammonia by a C-S-lyase present in the garlic plant termed alliin lyase or alliinase (Stoll and Seebeck, 1949 Helv Chim Acta 32: 197-205). Alliin and alliinase are found in different compartments of the garlic clove and the cutting or crushing of the clove releases the enzyme allowing it to come into contact with the precursor of allicin.

Allicin is unstable and breaks down into a number of different compounds some of which are thought to be of pharmaceutical use. A list of the various compounds present in garlic and their reported activity is given in Dr. Duke's Phytochemical and Ethnobotanical Database for Allium sativum. The main compounds of interest, in addition to allin and allicin, are the (E/Z)Ajoenes and the various dithiins.

Despite the impressive effect of garlic, studies have been limited by several factors such as lack of controlled methods and suitable double blind studies, and use of preparations with unknown amounts and chemical identification of the active agent. Allicin has been shown to exhibit the beneficial properties ascribed to garlic and thus it would be useful to be able to produce allicin in controlled and known amounts for use as the active ingredient of pharmaceutical compositions. However, allicin is a very labile and volatile compound when exposed to air and methods known today for its preparation are not satisfactory. The chemical synthesis involves many steps and is complicated, labourious, expensive, and very inefficient.

The use of an enzymatic method for producing allicin has been described as seeming to be "attractive", see WO97/39115. However, the so-called "suicidal" nature of the enzyme, namely that it is

rapidly and irreversibly rendered inactive by its own reaction product allicin, led the inventors of WO97/39115 to propose the use of an immobilized form of alliinase which is not inactivated by allicin.

The present invention offers an alternative method for the production of allicin, which is simple and inexpensive. By the use of the process of the invention it is possible economically to synthesis pure pharmaceutical grade allicin. The present invention also provides a method for the volume production of the ajoenes and dithiins as a consequence of being able to volume produce allicin. Previous methods have relied on the production of synthetic allicin.

According to a first aspect of the present invention, there is provided a method for preparing allicin which comprises the following steps:

(a) mechanically treating a natural source of alliinase to release alliinase therefrom;

(b) contacting the mechanically treated alliinase source with an aqueous solution of alliin containing alliin at a concentration greater than that found in raw garlic, whereby the alliin is enzymatically converted to allicin by the alliinase released from the alliinase source; and optionally

(c) extracting the resultant allicin into a low boiling point non-polar organic solvent.

Alliin exists in raw garlic at a concentration of around 0.4 to 0.9%, and varies significantly depending on the growth conditions of the garlic bulbs, which can vary from year to year.

Preferably, alliin is used in the method of the present invention at a minimum concentration of 1%, more preferably of 5%, more preferably still of 10% and most preferably at a minimum concentration of 15%.

Alliin is freely soluble in water and can therefore be used in the method of the present

invention at high concentrations. Preferably, alliin is used at a maximum concentration of 50%, more preferably of 40%, more preferably still of 30% and most preferably at a maximum concentration of 25%.

Preferably, alliin is used in the method of the present invention at a concentration of between 10% and 30%, more preferably at a concentration of between 15% and 25%. More preferably still, alliin is used in the method of the present invention at a concentration of around 20%. Use of alliin at a concentration of around 20% in the method of the present invention results in the production of an allicin solution with a concentration of around 1%.

Both the L form and the R form of alliin are converted to allicin by allinase. However, the L-form is converted at a slightly faster rate than the R-form.

The two forms of alliin require derivatising to be resolved. However, the presence of allicin in the reaction mixture interferes with the reaction using a derivatising agent. The observation that, as the conversion of alliin to allicin progresses, the HPLC alliin peak completely disappears, leads to the conclusion that both isomers of alliin are converted to allicin during the reaction. Further, under some conditions, the alliin peak appears as two partially resolved peaks, the sum of which is equal to traces showing only one peak. Fig. 4 shows a series of traces showing the growth of the allicin peak and the concurrent disappearance of the alliin peak

Step c), extraction using a low boiling point non-polar organic solvent is necessary in the preparation of pure allicin. However, in cases where some naturally occurring compounds other than allicin can be tolerated, the solution is diluted to approximately 1% allicin for storage at below -20°C, where it remains stable for several months. In the extraction step, the addition of a low boiling point non-polar solvent

prevents the presence of allicin leading to the denaturation of the allinase enzyme.

The extraction step has the advantage of purifying the allicin away from the crushed plant matter, thereby avoiding the potential presence of bacteria in the allicin solution. The extraction step also has the effect of slightly increasing the yield of allicin from a given amount of alliin and garlic.

The natural source of alliinase used in the process of the present invention may be matter obtained from an allium genus plant, typically the bulbous portion thereof. Most preferably it is garlic, Allium Sativum which is readily available and cheap and which also has a relatively high concentration of alliinase. However, any variety of garlic which contains sufficient quantities of allinase may be used, for example chinese garlic or elephant garlic. Fresh and/or dried garlic may be used, however, fresh garlic contains higher levels of alliinase. The natural alliinase source may be used in the invention in its raw form, although dry or frozen forms are also acceptable.

The natural plant source of alliinase, typically raw garlic bulbs or cloves which preferably will have been peeled and cleaned, is mechanically treated to release alliinase enzyme which is present within the structure of the natural alliinase source. This mechanical treatment may, for example, be crushing or cutting of the alliinase source. Other suitable methods which release the alliinase are also contemplated. One presently preferred method involves the use of a blender having a rapidly rotating blade which is able to disintegrate the alliinase source within a very short period of time. Any suitable industrial or domestic blender may be used. An advantage of blending is that the garlic cloves are reduced to very small pieces whilst the constituents of

the mixture are being thoroughly mixed.

Alternatively, the garlic could be crushed and then added to the alliin solution, followed by rapid stirring, However, this is not as efficient as the slow and continuous addition of garlic to the blender and produces a lower yield of allicin. It appears that the highest yields of allicin result from the addition of garlic at regular intervals combined with rapid blending and stirring. The allinase in the garlic reacts very quickly with the alliin before becoming inhibited by the presence of allicin, and the more allicin present, the greater the inhibition, as demonstrated by the reduction in reaction rate with solutions containing high levels of allicin, in Fig. 1.

Preferably, the production of allicin is carried out at a temperature of between about 25 and 45°C. More preferably, the production of allicin is carried out at a temperature of between about 30 and 40°C and most preferably at a temperature of 35°C.

The regular addition of garlic is preferably carried out over a period of at least one hour and more preferably over a period of between about 1 and 2 hours, and most preferably over a period of around 90 minutes.

The alliin solution may be made by dissolving crystalline alliin in water, preferably pure distilled water. Alternatively, the alliin can be formed in situ by oxidation, for example using hydrogen peroxide, of a solution of deoxyalliin of the appropriate strength (Iberyl *et al* (1990). *Plant Medica* **56**). The concentration of the alliin solution may preferably be up to 20% w/v. A typical concentration which may be used in the invention is about 10% w/v. Fig. 4 is a graph showing how the level of allicin produced from alliin varies depending on the level of alliin present in solution.

In accordance with the invention, the mechanically

treated alliinase source is contacted with an aqueous solution of alliin. This may be accomplished either by carrying out the mechanical treatment prior to the contacting step or, more preferably, by carrying out the mechanical treatment of the alliinase source within the alliin solution so that, when the alliinase is released, it is available for immediate reaction with the alliin. In this embodiment, where the natural source of alliinase is raw garlic cloves, the preferred mode of mechanical treatment is rapid blending which is preferably carried out for at least 30 seconds, preferably at least 1 minute and most preferably at least 5 minutes. In preferred aspects of this embodiment, the blending is continued throughout the whole of the contacting step.

In this embodiment, it is possible for the alliinase source such as garlic cloves to be added to the solution in more than one stage. Thus, after carrying out the contacting step for a first period of time, a further portion or portions of the alliinase source may be added to the solution and mechanically treated (e.g. blended with a blender) to release the alliinase therefrom, and the contacting step carried out for a second or further period of time. Preferably, the garlic is added slowly in several stages, for example, every 5 minutes, over a prolonged period of time, for example, over a period of up to 3 hours, more preferably less than 2 hours.

Where the mechanical treatment is carried out prior to the contacting step, it is in most cases necessary to bring the mechanically treated material into contact with the alliin solution within a relatively short period of time, before the released alliinase is significantly inactivated by allicin which is concurrently released with the alliinase. Typically, therefore, the material is added to the alliin solution and then immediately mechanically treated to release

the allinase and allow the released allinase to immediately contact the alliin. If the material is mechanically treated before contact with the alliin solution, any alliin present in the material may react with the allinase released by the mechanical treatment, to produce allicin which will inhibit the allinase enzyme.

The mechanically treated alliinase source is contacted with the alliin solution for a time and at a temperature such that alliin is converted, by the enzymatic action of the alliinase, to allicin. Thus, for example, the mechanically treated alliinase source may be contacted with the alliin solution for a period of time of from 2 to 4 hours and at a temperature of between about 20°C and about 40°C, preferably ambient temperature, i.e. about 25°C. A higher concentration of allicin is produced if the allinase source is added gradually over a period of time rather than the whole source of allinase being added at one time.

The amount of the alliinase source used is preferably that amount which is necessary to convert substantially all of the alliin in the solution to allicin. Where the alliinase source is garlic, it has been found that this can be achieved using an approximately equal weight of raw garlic to alliin in the solution. The alliin is converted to allicin, and the breakdown products of pyruvic acid, ammonia and carbon dioxide, with a conversion ratio for alliin to allicin of approximately 3:1. This is illustrated by the graph shown in Fig. 4. In addition, the graph of Fig. 3 shows the formation of pyruvic acid during the conversion of alliin to allicin.

The allicin formed in step (b) of the process of the invention is extracted into a low boiling point non-polar organic solvent. This extraction step may be carried out simultaneously with at least a part of the contacting step (b) or alternatively may be carried out

after completion of the contacting step. Thus, in one embodiment of the invention, the mechanically treated alliinase source (either previously prepared or formed in situ) is contacted with a mixture of the low boiling point non-polar solvent and the alliin solution. The solvent, which owing to its non-polar nature is not miscible with water, will form a separate layer. On blending, this is dispersed into small droplets throughout the alliin water layer. The allicin is soluble in the solvent layer and is immediately removed from the reaction thereby increasing the life of the enzyme to convert more alliin to allicin. The solution is allowed to separate into two layers and the allicin is recovered from the solvent by evaporation under vacuum to yield substantially pure allicin, which is then diluted and stabilized as required

In an alternative embodiment, the resultant product of step (b) containing formed allicin is combined with the organic solvent, whereupon extraction of the allicin into the solvent is accomplished. In this alternative embodiment, the resultant solution of step (b) of the process should preferably be contacted with the solvent immediately after completion of the contacting step, given the unstable nature of the allicin product.

Allicin is extremely unstable at concentrations above 0.5% solution in water. However, allicin is reasonably stable at a concentration of 0.5% solution at -40°C provided the solution has been filtered to remove impurities. The preferred concentration of allicin in solution after conversion from alliin is around 2%. Further, the stability of the allicin is increased in the presence of a slightly acidic solution. After extraction, with a solvent, to give pure allicin, the allicin must be kept at or below -70°C and breakdown of the allicin product begins immediately. Therefore, it is preferable to dilute the

allicin, in order to increase its stability, as quickly as possible, and preferably within 1 to 2 minutes, where possible. In this embodiment, the solvent will initially form a separate layer. The mixture may then be mixed thoroughly, for example by blending, so that the solvent is dispersed into small droplets throughout the alliin water layer. The allicin is soluble in the solvent layer and is immediately removed from the reaction. The solution is allowed to separate into two layers and the allicin is recovered from the solvent by evaporation under vacuum to yield substantially pure allicin, which is then diluted and stabilized as required.

It is possible, using the method of the invention, and if a sufficient quantity of the alliinase source is employed (in one or more stages if necessary), to convert most of the alliin present in the alliin solution to allicin. Fig. 2 is a graph showing the formation of allicin for a given amount of alliin with increasing addition of garlic. Under suitable HPLC conditions both alliin and allicin can be observed simultaneously allowing the conversion to be closely monitored. This enables the end point of the conversion to be accurately determined and so permits an operator of the process to properly time the next stage or stages of the process, bearing in mind the poor stability of allicin in solution. The conversion of alliin to allicin may be aided by providing a slight excess of alliin in the reaction solution.

In the method of the invention, the allicin is produced very rapidly, with the majority of the alliin typically being converted to allicin in under 30 minutes. The resulting process is thus suited to being carried out as a batch process with a short cycle time, which will produce a solution with a concentration of up to 1.0% v/v allicin. A solution of this strength is unstable. Accordingly, unless the allicin is extracted

into a solvent either in situ or immediately after completion of the contacting step, it should preferably be diluted to less than 0.5% w/v, for example about 0.15% w/v. The diluted solution may be further stabilized by adjusting the pH to about pH 4.5, using known method, for example a suitable buffer such as citric acid. Alternatively, the batch process can be adjusted to produce a solution with a concentration of up to 0.5% v/v allicin, which can be readily stored at -20°C.

In step (c) of the method of the invention, allicin produced by the reaction is extracted into a suitable non-polar solvent. The solvent used should have as low a boiling point as possible, preferably of around 45°C or lower, to allow rapid removal under vacuum at room temperature, be immiscible in water and have as low a water solubility as possible, whilst having the property of being capable of selectively dissolving allicin and no other constituent present in the solution. Examples of suitable solvents are pentane, hexane and ether. In the preparation of material for pharmaceutical use the choice of solvent is influenced by the EC guidelines relating to residual solvents (CPMP/ICH/283/95). These solvents are set out in MCA Euro Direct Publication No. 283/95 (see Appendix A), which lists 64 solvents with concentration limits and permitted daily intake, and these should be borne in mind when selecting a suitable solvent.

Step (c) of the method of the invention is particularly useful where pure allicin is required, for example, for the subsequent production of ajoene for pharmaceutical use.

The production of allicin from pure alliin and fresh garlic yields a material which is identical to that found in nature; any impurity such as pyruvic acid is found in nature as a result of the normal alliin reaction.

The present invention allows for the production of allicin in significant quantities and at strengths not previously attainable from raw garlic or other published or known method. In order to ensure the purity of the allicin product, a high purity starting material (alliin) should be used, either as a crystalline solid or high purity solution. The use of impure starting materials results in the product being coloured and greatly reduces the yield. Thus, it is a preferred aspect of the present invention that the alliin in step (b) is synthesised by a method in which pure deoxyalliin is oxidised using an oxidising agent such as hydrogen peroxide. Deoxyalliin may be synthesised using the method described by Iberl, Miller and Knobloch (1990) (Planta Med 56; 320-326), which method may be modified by the substitution of allyl chloride in place of allyl bromide. Special care should be given to control of the temperature throughout the reaction if the temperature, is too low, the reaction takes too long to complete and if the temperature is too high, the allinase enzyme activity is destroyed and any allicin produced rapidly breaks down. The present method ensures that the various impurities present as a result of the synthesis of allicin are avoided, in particular allyl chloride, acetic acid, sodium chloride and hydrogen peroxide at the relevant stages of the synthesis. For example, substituting allyl chloride for allyl bromide results in the production of sodium chloride rather than sodium bromide during the conversion process. It is also desirable to avoid the presence of excess hydrogen peroxide, if possible, as hydrogen peroxide has a tendency to explode during the crystallisation of alliin.

The allicin produced by this invention is substantially the same as naturally produced in garlic and may be used as a food additive or condiment, for

example to impart garlic flavour to oil, butter, cheese and the like or as a natural food preservative in the meat and milk industry.

The preparation of allicin in a pure and consistent form will enable it to be used for the manufacture of pharmaceutical compositions for human and veterinary use such as, but not being limited to viral, bacterial, fungal and parasitic infections, high levels of cholesterol and blood lipids, high blood pressure and thrombosis. These pharmaceutical compositions can be made by any standard method. The invention has the advantage of flexibility of solution concentration due to the high strength of the allicin solution produced which can then be diluted to any required concentration.

The pharmaceutical composition of the invention can be used in, but not limited to, the treatment of bacterial infections caused by bacteria of the genera Staphylococcus, Streptococcus, Vibrio and Bacillus, of fungal infections caused for example by Candida albicans, anti-amoebic and in the treatment of, for example, heart disease and arteriosclerosis. Recent studies have confirmed published reports on the antibacterial effectiveness of allicin.

In a further aspect of the invention the allicin is produced and extracted from the solvent layer and converted to ajoene using known methods (Iberl, Winkler and Knobloch, supra), which controls the conditions under which the allicin breaks down. The solution containing the allicin affects the breakdown product formed. 2-proponol or ethanol yields at least 60% (E/Z) ajoene. Specific control of the conversion conditions can yield a ratio of up to 90:10 of E ajoene :Z ajoene, or vice versa. Further, the production of 2-vinyl 1-2 diithin can be controlled via specific control of the conversion conditions.

The present invention provides a method for the

production of pure allicin and ajoene. These compounds, produced according to the method of the invention, yield compounds of a pharmaceutical grade compositions for the treatment for, but not limited to viral, bacterial, fungal and parasitic infections, high levels of cholesterol and blood lipids, high blood pressure and thrombosis.

In yet another aspect the invention provides substantially pure allicin or ajoene made in accordance with the method of the invention to be used as food additive, condiment or preservative.

Embodiments of the invention will now be described, by way of example only.

The present invention involves the conversion of pure alliin to allicin by the action of the enzyme alliinase present in, for example, fresh garlic. The allicin so produced can then be used as it is, extracted in a suitable solvent such as ether, pentane or hexane, to give pure Allicin or further processed to convert it to ajoene or diathiin.

In the examples, the following materials and methods are used.

Materials

L Cysteine was obtained from Forum Chemicals complete with a Kosher certificate of manufacture. Garlic was purchased from the local stores, as well as in bulk from wholesale markets and large scale importers. The imported garlic was from Spain and France. All other chemicals and solvents were purchased in the normal manner from usual UK Fine Chemical Suppliers.

HPLC

Analysis of the various compounds was carried out using a Hewlett Packard 1100 chromatograph fitted with an isocratic pump and variable wavelength detector. Chromatographic columns were Genisis C18 reverse phase, 250mm long x 4.6mm id, 5Á particle size, fitted with a

suitable guard column and supplied by Jones Chromatography. The mobile phase was 50% Methanol/50% water. The normal detection wavelength was 210nM.

Example 1

Method for the production of pure crystalline Deoxyalliin

The synthesis of Deoxyalliin was based on the method described by Iberl, Miller and Knobloch (1990) (Planta Med 56; 320-326) , but modified and substantially improved by the substitution of allyl chloride in place of allyl bromide, with special care being given to control of the temperature throughout the reaction.

2kg of L-cysteine hydrochloride monohydrate was dissolved in 2 litres of distilled water and stirred at 20-25°C. A solution of sodium hydroxide consisting of 1.6 kg in 2 litres of distilled water was added dropwise to the stirred reaction mixture over a period of 2 to 4 hrs. 1 litre of allyl chloride was then added slowly to the reaction mixture and the temperature was maintained between 25 and 30°C. To ensure completion, the reaction mixture was stirred for a further hour.

The reaction mixture was cooled to 4°C, then glacial acetic acid was added dropwise. During the addition of the acid, white solid started to separate out from the solution and the solution became very thick and difficult to stir. On completion of the addition of the acid the white solid was separated by filtration and dried.

Purification of the dried cake was carried out by dissolving the dried cake in 4 litres of distilled water, whilst maintaining the solution below 45°C. The purified deoxyalliin then began to crystallise and the mixture was cooled to -20°C to complete the crystallisation.

The solid was filtered off and dried. The resulting cake was washed 4 times with 0.5 litre of cold methanol at -20°C. After each washing the solid was dried to remove as much water as possible. The white solid was further washed 4 times with 5 litres of diethyl ether, and then dried, and finally the solid was dried under vacuum. The reaction yielded 1.61 kg of pure material, a recovery of between 90 and 99%.

Example 2

Method for the production of alliin

The deoxyalliin produced in the manner described above was used as the starting material for the production of alliin. Two different methods of alliin production are employed depending on the required end use of the alliin. The initial stages of the synthesis are identical. The deoxyalliin is dissolved and oxidised by hydrogen peroxide to form alliin. The control of the temperature is critical and the reaction rate and temperature is controlled by the rate of addition of the hydrogen peroxide. The alliin so produced is a racemic mixture of two isomers, as determined by the HPLC method of Iberl, Müller and Knobloch ((1990), supra) using a derivatising agent.

a) Alliin Liquid

6 litres of water was placed in a 10 litre round bottom flask and 1 kg of deoxyalliin was added while stirring. 500 ml of hydrogen peroxide (30% w/v) was added dropwise and the temperature maintained between 22 and 26°C during the addition. The reaction mixture was further stirred for 3 hours at room temperature to ensure completion of the reaction.

Deoxyalliin has a retention time of 3.4 minutes, L-cysteine 2.6 minutes, and alliin 2.9 minutes. This allowed the reaction to be followed in detail using HPLC to determine the completion point. A minimum quantity of hydrogen peroxide was used in the reaction and the alliin produced can be either used directly for

the production of allicin or crystallised to produce pure solid alliin.

b) Solid crystalline Alliin

1 kg of Deoxyalliin produced according to Example 1, was stirred in 1 litre water and 2 litre Methanol. 0.5 litres of Hydrogen peroxide was added dropwise while maintaining the temperature between 24-26°C. After the addition was complete the reaction mixture was further stirred for 4 hours to ensure completion of the reaction. The solution was refrigerated and cooled to -20°C to crystallise the Alliin. The white solid was filtered and washed 3 times with 1 litre of cold Methanol (-20°C). A total of 3 litres of Methanol was used to remove water. The white solid was further washed with 2 litres of Diethyl ether to remove traces of Methanol. The solid material was dried under vacuum to yield a weight of 0.95 kg.

Pure Alliin so produced has been used as a standard for the HPLC monitoring of the various reactions.

Example 3

Method for the production of an Allicin solution containing 1% Allicin, batch size 0.5 litre

Allicin is produced from the Alliin, prepared by the method detailed in (d), by the simple procedure of either dissolving in distilled water or taking a given amount of the liquid Alliin produced above and adding a small amount of fresh garlic in the form a pealed complete whole clove. The mixture is then combined in a blender for up to 5 minutes, typically 2 minutes, resulting in the complete disintegration of the garlic clove and the release of the garlic enzyme Alliinase. Conversion of the Alliin present by the enzyme alliinase occurs rapidly with approximately 50% of the Alliin being converted. Further additions of garlic result in a further conversion of Alliin to Allicin. The HPLC method described by Iberl, Müller and

Knobloch (1990) using a derivatising agent showed that both isomers of Alliin were converted to Allicin and substantially all Alliin present could be converted to Allicin. While the activity of the enzyme rapidly decreased following addition some activity remained for up to 24 hours, as demonstrated by Fig 1, with the Allicin increasing and the Alliin decreasing with time without further addition of the enzyme. Storage of the reaction mixture at 20°C did not completely stop the reaction.

The solution so produced can have a strength up to 2 % Allicin depending on the starting strength of the Alliin solution, which requires immediate dilution to below 0.5%, typically 0.15% (1500ppm), to render it stable. Control of the pH to approximately pH 4.5 increases the stability.

a) From solid crystalline alliin:

20 grams of L-Alliin was dissolved in 800ml of distilled water and 24 grams of Garlic cloves were added slowly over 3 hour period. The reaction mixture was filtered and 200 ml of water was added to bring the solution volume to 1 litre. This was stored at below +4 C.

b) From Deoxyalliin:

An alternative form of this invention is for the allicin produced by the action of the enzyme on alliin to be removed immediately on formation. This is achieved by the addition of a suitable immiscible organic solvent such as diethyl ether or hexane to the initial mixture before the addition of garlic and blending. On blending the immiscible solvent forms an emulsion with the alliin solution and the allicin as soon as its formed is absorbed by the tiny droplets of solvent so removing it from contact with the enzyme. The aim of this is to preserve and extend the activity of the enzyme so requiring the addition of less garlic. On completion of the conversion of the alliin to

allicin the reaction mixture is allowed to separate, the solvent containing the allicin removed and the pure allicin recovered as a straw coloured oily liquid by evaporation of the solvent under vacuum. Immediate dilution is required to prevent the breakdown of the allicin. 5grams of Alliin yield 1.5grams of pure allicin, a conversion ratio of 3.3 : 1.

50 grams of Deoxyalliin, produced according to method listed above was stirred in 225ml of distilled water and 25ml of hydrogen peroxide was slowly added. After 4 hrs the deoxyalliin was converted into alliin to yield a 14% (w/v) solution. This alliin solution was converted into 1% Allicin by the addition of whole garlic cloves, filtered and made up to 1.16 litres to yield a 1% solution as above.

Example 4

Method for the production of pure Allicin liquid

10grams of L-Alliin was added to 200ml of distilled water and 12 grams of fresh garlic cloves were added as described above in (Example 3). When the reaction was complete, after approximately 3 hrs., the reaction mixture was extracted with a suitable solvent such as 50ml of diethyl ether. The ether layer was separated, dried over Magnesium Sulphate, filtered and evaporated to a straw coloured liquid yielding 1 gram of allicin. This was dissolved in distilled water (100 ml) and the 1% Allicin solution was stored at -20 C.

Example 5

Method for the production of Ajoenes and Dithiins

1 gram of Allicin as prepared above (in Example 4) was dissolved in 10 ml of 40% water-acetone solution and the solution was heated between 63-64 C for 4 hours. The reaction mixture was diluted with 30ml of 50% water-methanol and washed 5 times with 10ml of n-pentane. The pentane was kept aside for further processing. The lower water-acetone-methanolic layer was then saturated with ammonium sulphate and extracted

with 20ml dichloromethane. The dichloromethane layer was separated, dried with Magnesium Sulphate, filtered and evaporated to yield crude Ajoene.

CLAIMS:

1. A method for preparing allicin which comprises the following steps:

(a) mechanically treating a natural source of alliinase to release alliinase therefrom;

(b) contacting the mechanically treated alliinase source with an aqueous solution of alliin containing alliin at a concentration greater than that found in raw garlic, whereby the alliin is enzymatically converted to allicin by the alliinase released from the alliinase source; and optionally

(c) extracting the resultant allicin into a low boiling point non-polar organic solvent.

2. A method according to claim 1 wherein the alliin is used at a minimum concentration of 1 %.

3. A method according to claim 2 wherein the alliin is used at a minimum concentration of 5 %.

4. A method according to claim 3 wherein the alliin is used at a minimum concentration of 10 %.

5. A method according to claim 4 wherein the alliin is used at a minimum concentration of 15 %.

6. A method according to any one of claims 1 to 5 wherein the alliin is used at a maximum concentration of 50 %.

7. A method according to claim 6 wherein the alliin is used at a maxium concentration of 40 %.

8. A method according to claim 7 wherein the alliin is used at a maximum concentration of 30 %.

9. A method according to claim 8 wherein the alliin is used at a maximum concentration of 25 %.

10. A method according to any preceding claim wherein the alliinase source is an allium genus plant.

11. A method according to claim 10 wherein the allium genus plant is *Allium sativum*.

12. A method according to any preceding claim wherein the mechanical treating in step (a) comprises crushing, cutting, or blending.

13. A method according to any preceding claim wherein in step (b), contacting the alliinase source with the aqueous source of alliin is carried out in several stages at regular intervals.

14. A method according to claim 13 wherein in step (b), contacting the alliinase source with the aqueous source of alliin is combined with rapid blending and stirring.

15. A method according to any preceding claim wherein at least step (b) is carried out at a temperature of between 25 °C and 45 °C.

16. A method according to claim 15 wherein at least step (b) is carried out at a temperature of between 30 °C and 40 °C.

17. A method according to claim 16 wherein at least step (b) is carried out at a temperature of 35 °C.

18. A method according to any preceding claim wherein step (b) is carried out over a period of at least 1 hour.

19. A method according to claim 18 wherein step (b) is carried out over a period of between 1 and 2 hours.

20. A method according to claim 19 wherein step (b) is carried out over a 90 minute period.

21. A method according to any preceding claim wherein the aqueous source of alliin is provided by dissolving crystalline alliin is distilled water.

22. A method according to any one of claim 1 to 20 wherein the aqueous source of alliin is provided by oxidation of a solution of deoxyalliin.

23. A method according to any preceding claim wherein the aqueous alliin solution is provided at a concentration of up to 20 % w/v.

24. A method according to claim 22 wherein the aqueous alliin solution is provided at a concentration of 10 %.

25. A method according to any preceding claim wherein step (c) is carried out after completion of step (b).

26. A method according to any one of claims 1 to 24, wherein step (c) is carried out simultaneously with at least a part of step (b).

27. A method according to any preceding claim wherein the concentration of allicin in solution after the completion of step (b) is 2 %.

28. A method according to any preceding claim wherein after completion of step (c), or step (b) if step (c) is not carried out, the allicin is diluted to a concentration of less than 0.5 % w/v.

29. A method according to claim 28 wherein after completion of step (c), or step (b) if step (c) is not carried out, the allicin is diluted to a concentration of 0.15 % w/v.

30. A method according to any preceding claim wherein the solvent used in step (c) is selected from the group consisting of pentane, hexane and ether.

31. A food additive or condiment comprising allicin produced by a method according to any preceding claim.

32. A pharmaceutical composition comprising allicin produced by a method according to any one of claims 1 to 30.

33. A pharmaceutical composition according to claim 32 for the treatment of bacterial infections caused by bacteria of the genera selected from the group comprising Staphylococcus, Streptococcus, Vibrio and Bacillus.

34. A pharmaceutical composition according to claim 32 for the treatment of fungal infections.

35. A pharmaceutical composition according to claim 32 for the treatment of heart disease and arteriosclerosis.

36. Use of allicin produced by a method according

to any one of claims 1 to 30 in the production of aojene.

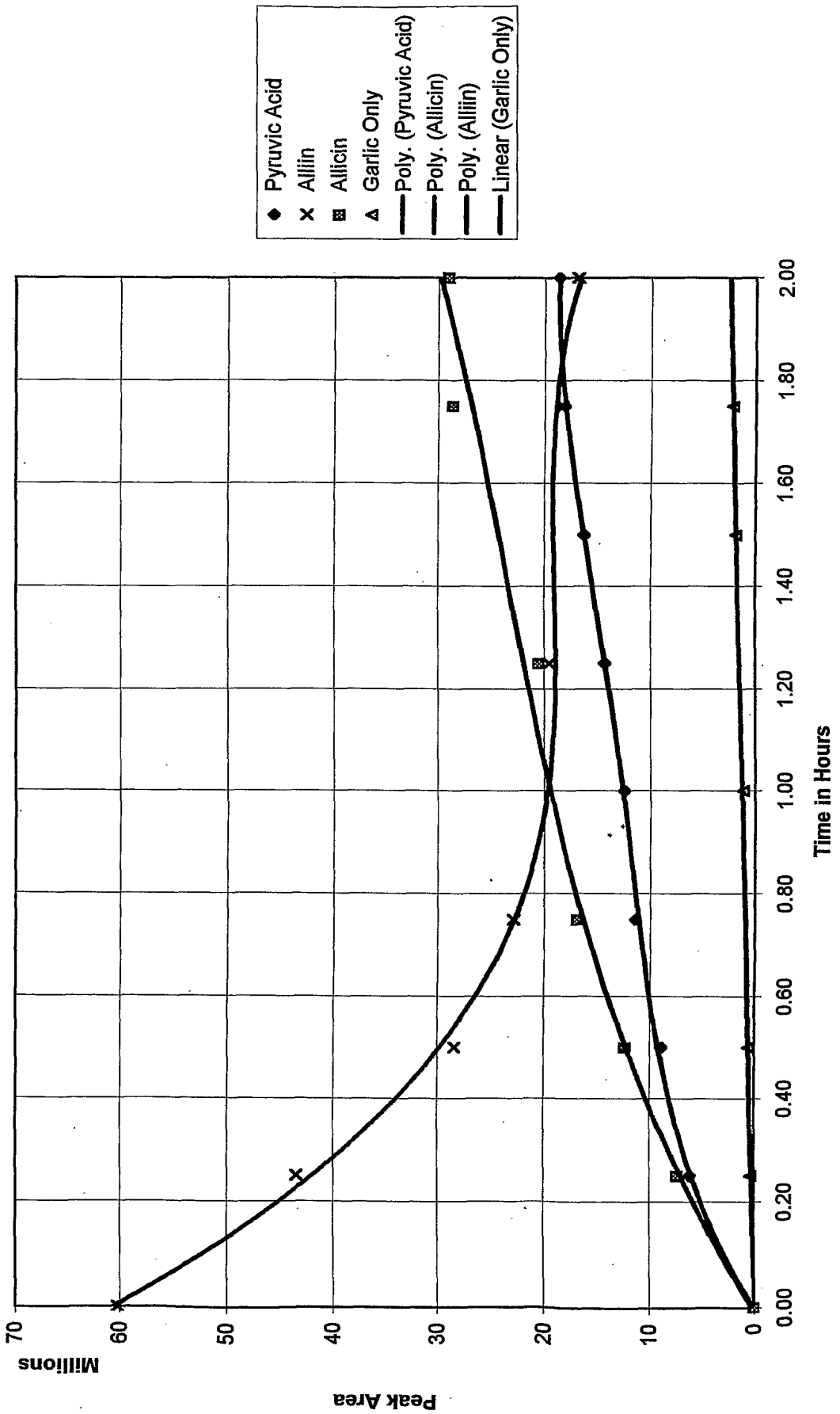

Fig 1 Alliin to Allicin Reaction

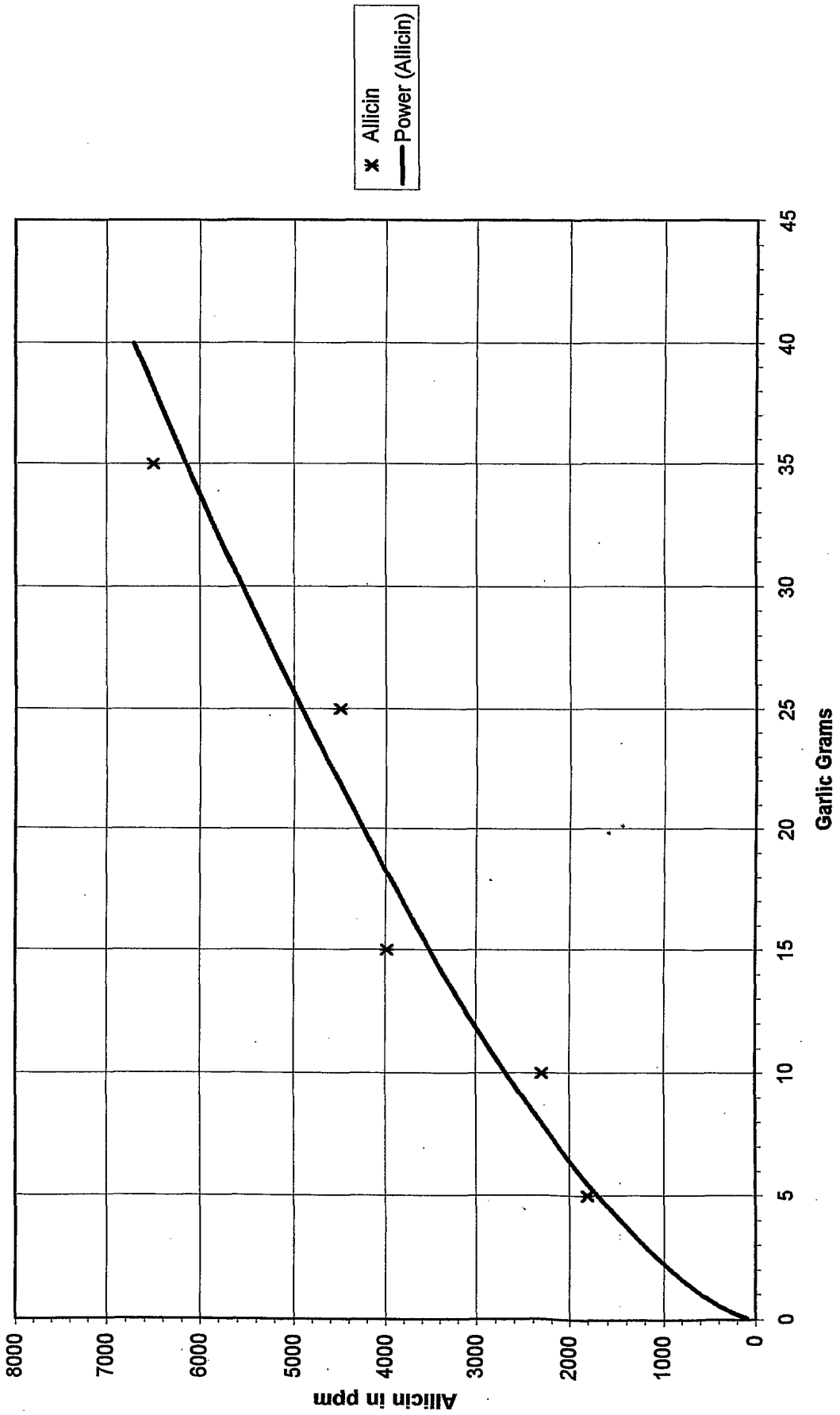

Fig 2 Formation of allicin

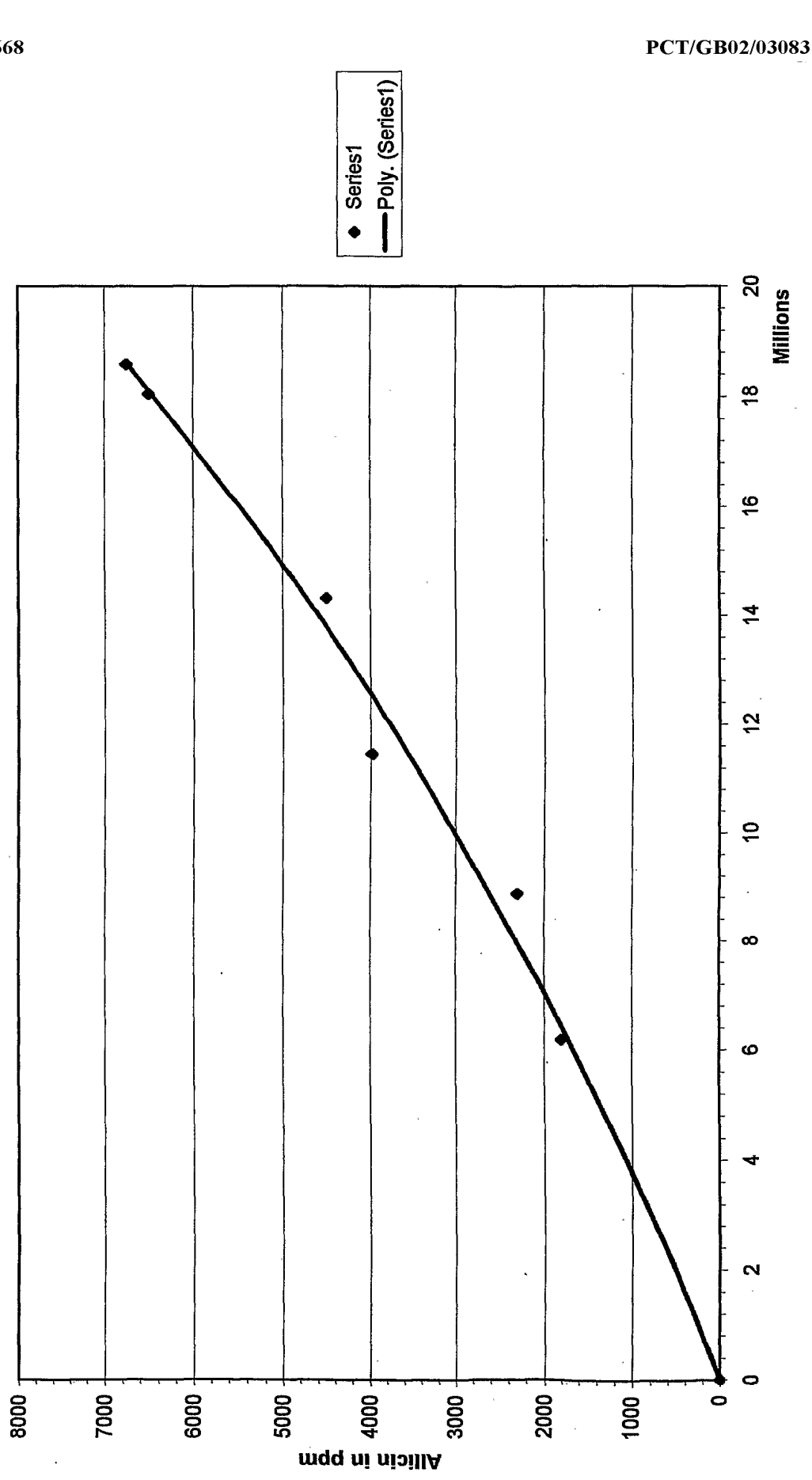

Fig 3 Pyruvic Acid vs Allicin

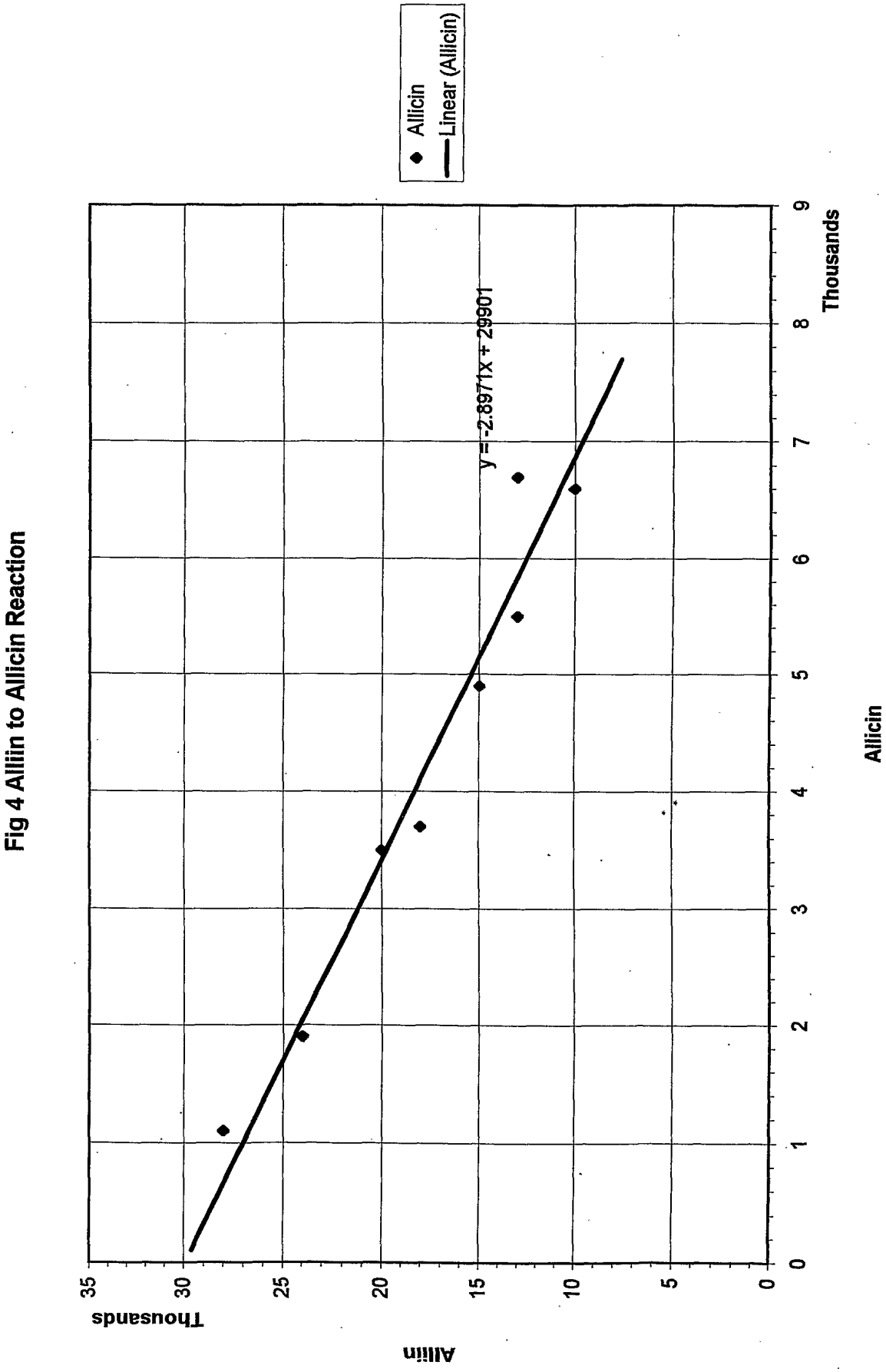

Fig 4 Alliin to Allicin Reaction

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/03083

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P11/00 A23L1/20 A61K31/255 A61P9/10 A61P31/04
A61P31/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 39115 A (MIRON TALIA ;MIRELMAN DAVID (IL); RABINKOV AHARON (IL); WILCHEK ME) 23 October 1997 (1997-10-23) cited in the application page 7, line 11 -page 8, line 2 page 9, line 25 -page 10, line 2 example 4 claims 11-15 ---	1-35
A	US 5 741 932 A (PRIGGE HELMUT ET AL) 21 April 1998 (1998-04-21) column 2, line 9 - line 63 column 4, line 6 - line 16 claims 1-6 -----	36

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 2002	17/12/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bayer, A

Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-30 (all partially)

Present claims 1-9,12-30 relate to a natural source of aliinase withouth further specification and present claims 1-29 refer to an organic solvent defined by reference to a desirable characteristic, namely its low boiling point.
The claims cover all natural sources of alliinase and all organic solvents having this characteristic , whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such natural sources and solvents. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the natural source and the solvent by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to allium sativum as natural source (see page 5 lines 9-12, claim 10,11) and the organic solvents pentane, hexane and ether mentioned in the description at page 11 lines 20,21 and in claim 30.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/03083

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-30 (all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/03083

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9739115 A	23-10-1997	AU 2305897 A	07-11-1997
		CA 2251532 A1	23-10-1997
		EP 0904361 A1	31-03-1999
		WO 9739115 A1	23-10-1997
		JP 2000508535 T	11-07-2000
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		DE 59603938 D1	27-01-2000
		EP 0721940 A1	17-07-1996
		JP 2820653 B2	05-11-1998
		JP 8259525 A	08-10-1996